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DOI:

[10.1016/j.jaut.2016.04.008](https://doi.org/10.1016/j.jaut.2016.04.008)

*Document Version*

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*Citation for published version (APA):*

Bertolaccini, M. L., Contento, G., Lennen, R., Sanna, G., Blower, P. J., Ma, M. T., Sunassee, K., & Girardi, G. (2016). Complement inhibition by hydroxychloroquine prevents placental and fetal brain abnormalities in antiphospholipid syndrome. *Journal of Autoimmunity*, 75, 30-38. <https://doi.org/10.1016/j.jaut.2016.04.008>

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**Title:** Complement inhibition by hydroxychloroquine prevents placental and fetal brain abnormalities in antiphospholipid syndrome. Maria Laura Bertolaccini<sup>1</sup>, Gregorio Contento<sup>1</sup>, Ross Lenner<sup>2</sup>, Giovanni Sanna<sup>3</sup>, Philip J Blower<sup>4</sup>, Michelle Ma<sup>4</sup>, Kavitha Sunassee<sup>4</sup>, Guillermina Girardi<sup>1,5</sup>.

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## CONFLICT OF INTEREST STATEMENT

The authors have nothing to declare.

## Word count:

## ABSTRACT

Placental ischemic disease and adverse pregnancy outcomes are frequently observed in patients with antiphospholipid syndrome (APS). Despite the administration of conventional antithrombotic treatment a significant number of women continue to experience adverse pregnancy outcomes, with uncertain prevention and management. Efforts to develop effective pharmacological strategies for refractory obstetric APS cases will be of significant clinical benefit for both mothers and fetuses. Although the antimalarial drug, hydroxychloroquine (HCQ) is increasingly used to treat pregnant women with APS, little is known about its efficacy and mechanism of action of HCQ.

Because complement activation plays a crucial and causative role in placental ischemia and abnormal fetal brain development in APS we hypothesised that HCQ prevents these pregnancy complications through inhibition of complement activation. Using a mouse model of obstetric APS that closely resembles the clinical condition, we found that HCQ prevented fetal death and the placental metabolic changes - measured by proton magnetic resonance spectroscopy in APS-mice. Using <sup>111</sup>In labelled antiphospholipid antibodies (aPL) we identified the placenta and the fetal brain as the main organ targets in APS-mice. Using this same method, we found that HCQ does not inhibit aPL binding to tissues as was previously suggested from *in vitro* studies. While HCQ did not affect aPL binding to fetal brain it prevented fetal brain

abnormal cortical development. HCQ prevented complement activation *in vivo* and *in vitro*. Complement C5a levels in serum samples from APS patients and APS-mice were lower after treatment with HCQ while the antibodies titres remained unchanged. HCQ prevented not only placental insufficiency but also abnormal fetal brain development in APS. By inhibiting complement activation, HCQ might also be an effective antithrombotic therapy.

## **KEYWORDS**

Animal model - Antiphospholipid syndrome - Pregnancy – hydroxychloroquine -  
Proton magnetic resonance spectroscopy - SPECT/CT

## INTRODUCTION

The antiphospholipid syndrome (APS) is an autoimmune disease associated with the presence of antiphospholipid autoantibodies (aPL). APS occurs most commonly in young women of reproductive age and is characterised by adverse pregnancy outcomes frequently associated with placental pathologies [1-4].

There is growing evidence for transplacental passage of aPL, and brain abnormalities and cognitive impairment has been described in infants born to mothers affected by APS, suggesting that exposure to aPL *in utero* can affect fetal brain development and thus might induce behavioural and cognitive problems later in life [5-7]. Abnormal behaviour in the offspring of APS-mice has also been described [8, 9]. Thus, the impact of maternal aPL antibodies on the offspring may extend beyond the known association with adverse pregnancy outcome.

aPL constitute a heterogeneous group of autoantibodies with different specificities and different target organs' principally the female reproductive system and the vascular system.  $\beta_2$ -glycoprotein I ( $\beta_2$ GPI) is the major antigen for clinically relevant antibodies in APS [4]. *In vitro* studies demonstrated that a $\beta_2$ GPI antibodies isolated from patients avidly bind to trophoblast monolayers isolated from human placentas [10, 11] and to isolated mouse fetal cortical neurons [9]. Furthermore, some of these *in vitro* studies demonstrated that a $\beta_2$ GPI affect trophoblast function and invasion [12, 13] and mouse fetal cortical neurons cytoarchitecture [9]. Ex-vivo studies also showed robust aPL deposition in placentas in mice and humans [9, 14, 15]. However, no data are available on the binding of a $\beta_2$ GPI antibodies and the identification of the target organs *in vivo*.

Pregnancy complications in APS have been attributed to placental thrombosis and infarcts and management of obstetric APS is based on attenuating the

procoagulant state. However, in many cases there is no evidence of decidual thrombosis or placental vasculopathy, and instead inflammatory signs are present [16-18]. Treatment with aspirin and heparin has become a conventional option for women with obstetric APS. While the use of aspirin and heparin has improved the pregnancy outcome in obstetric APS, current treatment fails in a significant number of pregnancies [19, 20] raising the need to explore other treatments to improve obstetrical outcome.

Hydroxychloroquine is a medication originally used to prevent/treat malaria. This antimalarial drug has also been used to treat pregnant women with APS for many years. While recent studies suggest that HCQ might improve pregnancy outcomes in APS [21], the use of HCQ is still controversial; most of the beneficial effects of HCQ in pregnancy constitute anecdotal evidence and little is known about its mechanism of action [22-24].

Here we evaluate the effects of HCQ in the mother and developing fetus in a mouse model of obstetric APS. Knowing that complement activation plays a crucial role in the pathogenesis of adverse pregnancy and fetal outcomes in APS in mice and women [9, 14, 17, 25-27], we hypothesised that HCQ protects pregnancy in APS by inhibiting complement activation.

## **METHODS**

### **Purification of aPL antibodies**

anti $\beta_2$ GPI antibodies were isolated from 7 patients with primary APS (PAPS).

Autoantibody profiles and clinical features from patients (untreated and treated with HCQ) are described in **Table 1**. All patients met Sidney Laboratory criteria for APS [28]. The patients were identified through the Registry of Connective tissue diseases

(10/H0405/35) at St Thomas' Hospital. The NHS National Research Ethics Service approved the collection and utilization of samples for research purposes.

IgG from APS patients and from healthy non-autoimmune individuals was purified using protein G sepharose chromatography [17]. A further affinity purification step using peptides mimetic to regions of  $\beta_2$ GPI immobilised onto magnetic beads was performed in the IgG fractions from patients with APS in order to obtain purified  $\alpha\beta_2$ GPI antibodies [29]. Blank et al. demonstrated that these peptides – that correspond to three epitopes located in domains I-II, III and IV of the  $\beta_2$ GPI molecule – bind to human anti- $\beta_2$ GPI Abs and prevent fetal loss and endothelial activation in experimental APS [29]. Functionality of the isolated  $\alpha\beta_2$ GPI antibodies was confirmed by ELISA. Endotoxin removal in each sample was performed using high capacity endotoxin removal spin columns (Pierce Thermoscientific). All samples showed to be endotoxin free ( $< 0.01$  ng/mL) using the LAL Chromogenic Endotoxin Quantitation Kit.

## **Animals**

All housing and experimental procedures were performed in compliance with the UK Home Office Animals Scientific Procedures Act 1986 (Home Office project licence number 60/4305). C57BL/6 mice (2-3 months old) purchased from commercial vendors were used in all experiments. A group of females were mated with previously isolated males. The presence of a vaginal plug defined day 0 of pregnancy.

## **Mouse model of obstetric APS (APS-mice)**

Affinity purified antibodies to  $\beta_2$ GPI isolated from patients and normal human IgG (NHlgG) (control antibodies) were administered intravenously by a bolus injection (100  $\mu$ g/mouse) on day 7 of pregnancy followed by maintenance doses through a microosmotic pump ( Alzet, model 1002, pumping rate 0.25  $\mu$ l/h, reservoir volume 100  $\mu$ l). This model uses similar amounts of aPL antibodies to the passive transfer model considering that in the passive transfer model the whole IgG fraction from patients with APS are injected intraperitoneally [17]. Administration through microosmotic pumps ensures constant antibody concentrations are maintained throughout pregnancy to closely resemble the clinical condition. A group of pregnant mice received hydroxychloroquine (HCQ, dissolved in sterile distilled water) administered by a second microosmotic pump (Alzet model 1002) inserted on day 8 of pregnancy. Based on clinical treatment regimes, in which the recommended dosage is 6.5 mg/kg/day, we studied the effects of 200  $\mu$ g HCQ/mouse/day.

### **Single photon emission computed tomography (SPECT/CT) imaging of $\alpha\beta_2$ GPI biodistribution *in vivo***

#### *Preparation of $^{111}\text{In}$ -labelled antibodies.*

Antibodies to  $\beta_2$ GPI and control antibody NHlgG were conjugated with the bifunctional chelator p-SCN-Bn-CHX-A''-DTPA -using the same chelator/antibody ratio- as described previously [30]. The immunoconjugates were then isolated in ammonium acetate solution (0.2 M, pH 6) by purification with size exclusion columns and radiolabelled with an equal volume of  $^{111}\text{InCl}_3$  (Mallinckrodt Medical B.V., Petten, Netherlands). The radiolabelled conjugates were isolated using an a HPLC BioSep SEC s2000 size exclusion column (Phenomenex, Macclesfield, UK) (isocratic mobile



phase, PBS containing 2 mM EDTA, 1 ml/min, UV detector:  $\lambda = 280$  nm).

Radiochemical yield and purity was > 97%. Day 13 pregnant and non-pregnant mice were injected intravenously were injected intravenously with 15-35 MBq  $^{111}\text{In}$ - $\alpha\beta 2\text{GPI}$  antibodies (80  $\mu\text{L}$ , 0.56 mg  $\text{mL}^{-1}$ , 45  $\mu\text{g}$  of immunoconjugate) and scanned 48 h later (predetermined maximal binding) at gestational day 15. Images to visualize IgG binding to different target organs were captured under anaesthesia using a NanoSPECT/CT Silver Upgrade preclinical scanner (Mediso Ltd., Budapest, Hungary). After whole body imaging the mice were killed and organ/tissues were dissected and frozen. The net counts per minute (CPM) for each tissue and standard were determined using a Wallac 1282 Compugamma Universal Gamma Counter. The percent injected radioactivity dose (%ID) was determined from CPM in the tissue calibrated against a sample counted in the dose calibrator used to measure the injected dose. The content of radiolabelled IgG in each tissue was expressed as ng IgG per gram of tissue calculated from the %ID/g and the specific activity of the labelled antibody.

### **Proton Magnetic resonance spectroscopy studies ( $^1\text{HMR}$ S)**

This non-invasive *in vivo* imaging modality was used to study the biochemical and metabolic profile in placentas and fetal brains in APS-mice and respective controls treated with NHlgG. All MRI experiments were performed using a 7-Tesla horizontal bore NMR spectrometer (Agilent, Yarnton, UK), equipped with high-performance gradient insert (12 cm inner diameter), maximum gradient strength 400 mT/m). At day 15 of pregnancy, APS and control mice were anaesthetized with 1.8% isoflurane in oxygen/air (50/50, 1L/min) and placed in a cradle (Rapid Biomedical GmbH, Rimpar, Germany). Spectra were analyzed using LCModel (<http://s->

[provencher.com/pages/lcmodel.shtml](http://provencher.com/pages/lcmodel.shtml). The  $^1\text{H}$ -MRS signal from creatine (Cr) (measure of the global brain function or integrity) was used to normalize the signals from other metabolites measured within the same voxel.

### **Immunofluorescence studies**

Frozen sections from placentas and fetal brains from control mice were stained with antibodies to  $\beta 2\text{GPI}$  (Santa Cruz, apoH antibody (M-190):sc366275, dilution 1/400) followed by FITC-conjugated anti rabbit IgG (Sigma-Aldrich F9887, dilution 1/400) to investigate the presence of the protein in these tissues. Frozen sections from placentas and fetal brains from mice treated with  $\alpha\beta 2\text{GPI}$  and  $\alpha\beta 2\text{GPI}$  +HCQ-treated mice were stained for  $\alpha\beta 2\text{GPI}$  deposition using a Texas Red-conjugated anti human IgG antibody (Thermo Scientific, PA1-28834, dilution 1/500). Texas Red-conjugated anti human IgG antibody was also used to visualize NHlgG in placenta and fetal brain in NHlgG-treated mice. Antibodies to microtubule associated protein-2 (MAP-2; Sigma-Aldrich M3696, dilution 1/100) - a marker of intact neuronal cell bodies - and neurofilament 200 (NF-200; Sigma Aldrich N4142, dilution 1/400) – marker of dendritic and axons structure- were used to investigate neurons cytoarchitecture [9]. Bound antibodies were detected using a FITC-conjugated anti rabbit IgG (Sigma-Aldrich F9887, dilution 1/400). Frozen sections of placentas and fetal brains were stained for complement deposition, using monoclonal anti-C3b, iC3b, C3c antibodies (clone 2/11, Hycult Biotech, dilution 1/50) followed by a FITC-conjugated anti-rat IgG (Sigma-Aldrich F6258, dilution 1/400). Diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, 62248) was used for nuclear counterstains in all immunofluorescence studies.

Production of superoxide anion in placentas was measured using dihydroethidium (DHE, Sigma-Aldrich 37291).

### **ELISA to evaluate the effect of HCQ on antibody complement fixing and activating capacity**

Complement fixing capacity was measured by an ELISA that uses mouse aPL antibodies (FB1, mouse IgG2b) which have been shown to activate complement in different mouse models [31, 32]. Non-fixing antibody FD1 was used as control [32]. Polystyrene plates were coated with  $\beta$ 2GPI and cardiolipin. FB1 or control antibody FD1(100  $\mu$ g/ml) were incubated onto the polystyrene plates. After washing with PBS/tween, a source of complement (normal mouse serum, dilution 1/100) was added to the wells with and without increasing concentrations of HCQ (20-50 and 100 ng/ml). HCQ concentrations were calculated based on the clinical recommended dose (6.5 mg/kg) and blood volume of distribution of 116 L/kg [33]. Plates were incubated 30 min at 37°C under continuous shaking. The wells were aspirated and stored at -°20C for the determination of complement C3 split product C3adesArg by ELISA and washed.

To study C1q binding, antimouse C1q-HRP was added and incubated for 1h RT, then washed as described before. To study C3 activation, monoclonal rat antibody anti mouse C3b/iC3b/C3d (Hycult, USA) was added and incubated for 1 h RT, then washed as described before. Secondary antibody (anti-rat IgG H&L-HRP) was added and incubated for 1h at RT, then washed as above. HRP was developed using prewarmed substrate (TMB). Assays were performed in duplicate and read at 450nm. Background was assessed as the binding to wells without any  $\beta$ 2GPI/cardiolipin added and was subtracted from the coated plates. Other controls

consisted of mouse serum from C3 deficient mice and C1q deficient mice instead of fresh mouse serum.

### **Measurement of C3a and C5a**

The C3adesArg and C5adesArg in serum and wells contents from the complement fixing capacity ELISA were measured by sandwich enzyme-linked immunosorbent assay (ELISA) as previously described [34].

### **Statistical analysis**

Data are expressed as mean  $\pm$  SD. Statistical differences between groups were determined using one-way ANOVA with subsequent two-tailed Student t test.

## **RESULTS**

### ***HCQ prevented fetal death and placental abnormalities in APS***

Continuous exposure to aPL antibodies through microosmotic pump increased the fetal resorption frequency compared to the passive transfer model in which antibodies are given in two single injections [17]. In the new APS-model more than 50% of the embryos did not survive (Figure 1A, **Table 2**). The surviving fetuses were growth restricted and placentas were smaller compared to control NHIgG-treated mice (**Table 2**). Increased production of superoxide anion –indicative of increased oxidative stress was observed in placentas from  $\alpha\beta 2$ GPI –treated mice (**Figure 1B**). Administration of HCQ to APS-mice prevented fetal death, increased placental and fetal weight and decreased placental superoxide production (**Table 2, Figure 1A**). HCQ-treated mice showed normal pregnancy outcomes, comparable to NHIgG-treated mice (**Table 2**).

<sup>1</sup>H-MRS was used to assess the metabolic profile of placentas *in vivo* in utero at day 15 of pregnancy. The <sup>1</sup>H-MRS signal from creatine (measure of the global brain and placental function or integrity) was used to normalize the signals from other metabolites measured within the same voxel. A significant decrease in chemical mediators glucose (Glc/Cr), lactate (Lac/Cr) and choline derivatives (GPC/Cr and PCh/Cr) - involved in energy metabolism, membrane lipid function and neuroprotection – was observed in placentas from APS-mice compared to NHlgG-treated mice (**Table 3**). HCQ prevented these placental metabolic abnormalities in APS-mice (**Table 3**)

#### ***HCQ protects fetal brain development***

Diminished NF200 (marker of dendritic and axons structures) and MAP-2 (marker intact neuronal cell bodies) staining - indicative of abnormal neuronal cytoarchitecture - was observed in the fetal brains in APS-mice compared to NHlgG-treated mice ([9], **Figure 1C**). HCQ prevented aPL-induced abnormal fetal cortical brain development (**Figure 1C**). Similar to what was observed in the placenta, a significant decrease in Glc/Cr, Lac/Cr, GPC/Cr and PCh/Cr was also detected in fetal brains from APS-mice (**Table 3**). HCQ prevented these metabolic abnormalities in fetal brains in APS-mice.

#### ***aPL binding in vivo - SPECT/CT studies***

aβ<sub>2</sub>GPI antibodies and NHlgG were labelled with <sup>111</sup>In and visualized *in vivo* using SPECT/CT in pregnant and non-pregnant mice (see videos in Suppl Material). In the pregnant mouse <sup>111</sup>In -aβ<sub>2</sub>GPI were rapidly cleared from the circulation and deposited in the fetal sacs (**Figure 2B left, 2B1**). Within the fetal sacs <sup>111</sup>In -aβ<sub>2</sub>GPI

antibodies were detected in placentas and fetal brains by ex-vivo gamma counting (**Table 4**). In contrast to pathogenic  $\alpha\beta_2$ GPI, a large amount of the injected dose of  $^{111}\text{In}$ -NHlgG remained in circulation at 48 hours (**Table 4**). SPECT/CT studies showed the presence of  $^{111}\text{In}$ -NHlgG in the placenta but not in the fetus (**Figure 2B right, 2B2**). Ex-vivo gamma counting, showed minimal amount of  $^{111}\text{In}$ -NHlgG in the fetal brains (**Table 4**). In the non-pregnant mouse  $^{111}\text{In}$ - $\alpha\beta_2$ GPI and  $^{111}\text{In}$ -NHlgG remained longer in circulation compared to pregnant mice (**Figure 2A, Table 4**).  $\alpha\beta_2$ GPI antibodies were found in the spleen and liver of the non pregnant mouse (**Figure 2A left**).

Immunohistochemical studies confirmed the presence of  $\alpha\beta_2$ GPI antibodies in the placenta (**Figure 3A**) and fetal cortical brain detected by SPECT/CT and ex-vivo gamma counting (**Figure 3B**). In accordance with the ex-vivo gamma counting studies, NHlgG deposition was found in the placenta but not in the fetal brain (Figure 3C and D).

Staining for  $\beta_2$ GPI in placentas and fetal brains showed abundant expression of the protein (**Figure 3E and 3F**) that might explain the tropism of  $\alpha\beta_2$ GPI for these organs.

### ***HCQ does not affect aPL binding to placenta and fetal brain in pregnant mice***

Because a recent *in vitro* study suggested that HCQ reduces the binding of aPL antibodies to the syncytiotrophoblast [35, 36], we investigated if this mechanism could account for the protective effects of HCQ we observed in obstetric APS in mice *in vivo*.

Ex-vivo gamma counting in pregnant mice demonstrated that the administration of HCQ does not affect the binding of  $^{111}\text{In}$ - $\alpha\beta_2$ GPI to placentas and fetal brain (**Table**

4). The biodistribution of  $\alpha\beta_2$ GPI in pregnant mice treated with or without HCQ was identical, the placentas and fetal brains the main target sites (**Table 4**).

Immunohistochemical studies in placentas and fetal brain confirmed the SPECT/CT and ex-vivo gamma counting observations (**Figure 4A**). Staining for  $\alpha\beta_2$ GP antibodies in  $\alpha\beta_2$ GP+HCQ-treated mice was not different from mice treated with  $\alpha\beta_2$ GP alone (**Figure 4A**).

#### ***HCQ inhibits antibody complement fixing and activating capacity in vitro***

To investigate the effects of HCQ on antibody complement fixation and activation capacity we used an ELISA method. Addition of HCQ inhibited the binding of C1q to mouse complement fixing antibody FB1 and also prevented generation of complement split products C3a and C3b/iC3b/C3d (**Table 5**). A dose response effect was observed with total inhibition at a concentration of HCQ of 100 ng/ml (**Table 5**). When serum from mice injected with HCQ was used as a source of complement, complement C1q fixing capacity and C3 activation was also abolished (**Table 5**).

#### ***HCQ inhibits complement activation in vivo***

##### **Mouse studies**

Increased complement deposition that was previously described in placentas and fetal brains in the mouse model of APS were not observed when mice received HCQ ([9], **Figure 4B**). In addition, complement split products C3a and C5a levels decreased dramatically in  $\alpha\beta_2$ GPI treated with HCQ, compared to mice that received only the pathogenic antibody (**Table 6**).

##### **Human studies**

To investigate if complement inhibition occurs after treatment with HCQ in humans, we measured complement activation product C5adesArg levels and antibody titres in serum from APS patients before and after treatment with HCQ (6.5 mg/kg, >6 months after starting the treatment). C5adesArg levels diminished after treatment with HCQ while the aCL and a $\beta$ 2GPI titres remained unchanged (**Table 7**).

## DISCUSSION

Placental-mediated pregnancy complications are frequently associated with antiphospholipid antibodies. While several *in vitro* studies demonstrated binding of aPL antibodies to trophoblast cell lines and primary human trophoblasts [10-13] and fetal cortical neurons *in vitro* [9] it is still unclear if this happens *in vivo* and what pathogenic mechanisms play a role. Here we found evidence that aPL antibodies bind to the placenta and fetal brain *in vivo*. Using *in vivo* SPECT/CT we found that a $\beta$ 2GPI antibodies are rapidly cleared from circulation and large amounts of the radiolabelled antibody are entrapped in the placentas and fetal brains within the fetuses in the fetal sacs. To our knowledge this is the first study to show *in vivo* binding of antibodies to the placenta and the developing fetal brain. This study demonstrates that pathogenic antibodies to  $\beta$ <sub>2</sub>GPI are capable of crossing the placenta, the blood brain barrier (BBB) and binding to the fetal brain. Direct binding of aPL antibodies to the developing brain might explain the abnormal fetal brain development and abnormal behaviour that we and others reported in the offspring of APS-mice [8, 9]. In humans, a prospective multicentre registry of a cohort of children born to mothers with APS showed presence of neurodevelopmental abnormalities [6]. The placenta was also a hot spot in mice injected with radiolabelled NHIgG, this can be explained by the abundant expression of the neonatal FcRn receptor that



facilitates the transfer across the placenta of normal IgG [37].

Despite the lack of knowledge about its efficacy and mechanism of action, HCQ has been used to treat pregnant women with APS for many years now [21-24]. HCQ protected pregnancies in the mouse model of APS; fetuses survived and normal placental and fetal brain development was observed in APS-mice treated with HCQ. In addition, HCQ prevented the metabolic abnormalities observed in the placenta and fetal brains in APS-mice. Despite the known associations between placental dysfunction and fetal brain development, there are few available tools to evaluate *in vivo* the placental metabolism and function during pregnancy. In this study, we used <sup>1</sup>HMRS as an important tool to determine *in vivo* metabolism [38]. This non invasive methodology might have significant potential clinical implications, such as the opportunity to identify APS patients at risk of placental and fetal brain abnormalities and the use of preventive therapy. Deficiency in chemical mediators involved in energy metabolism, membrane lipid function and neuroprotection were found in placentas and fetal brains in APS-mice. HCQ reversed these abnormal metabolic profiles. Oxidative stress affects placental metabolism and transport of metabolites to the fetus [39, 40]. Increased reactive oxygen species production induced by a $\beta_2$ GPI antibodies in placentas from APS-mice, can affect placental metabolism and transport of nutrients to the fetus contributing to abnormal fetal brain development. Because low levels of choline have also been associated with abnormal trophoblast function [41] and choline is a neuroprotectant [42], we can speculate that deficiency of choline not only affects placental function but might also exacerbate the direct neurotoxic effects of a $\beta_2$ GPI antibodies.

While the use of HCQ appears to be safe in women little is known about its mechanism of action [43-45]. While some *in vitro* studies suggested that HCQ

inhibits aPL binding to trophoblasts restoring their function [35, 36], our ex vivo gamma counting and immunohistochemical studies demonstrated that HCQ at a dose similar to the therapeutic dose in human, does not affect the binding of aPL to either the placenta or the fetal brain.

An association between complement activation and placental insufficiency and abnormal fetal brain development was observed in mice treated with a $\beta$ <sub>2</sub>GPI antibodies. We previously demonstrated using MRI that complement deposition is observed in fetal brain and placenta in APS mice and this predicts adverse outcomes [9]. HCQ was shown to inhibit complement activation *in vivo* and *in vitro*. The *in vitro* studies suggest that HCQ inhibits complement activation by inhibiting the binding of C1q, the first step in the activation of the classical pathway. By inhibiting C1q binding, HCQ also inhibits the generation of the C3 convertase and cleavage of C3. Our studies do not rule out that HCQ might also inhibit complement activation by other pathways downstream of C3. That HCQ inhibits complement activation explains why while aPL antibodies are found in placenta and fetal brain in APS-mice treated with HCQ, no pathogenic effects were observed in these tissues. Decreased plasma complement split product levels and diminished C3b deposition were observed in placentas and fetal brains in APS-mice treated with HCQ and this was associated with improvement in placental metabolism, diminution of oxidative stress and prevention of abnormal neurodevelopment. These data suggest that complement inhibition contributes to the protective effects of HCQ on fetal brain and placenta in obstetric APS.

Inflammation is thought to play a crucial role in the pathogenesis of APS [46, 17]. It has been reported that HCQ decreases leukocyte activation and reduces cytokine and reactive oxygen species production [47, 48]. Knowing that C5a is a

potent activator of inflammatory cells, it is tempting to speculate that the effects of HCQ in reducing inflammatory cells activation can be mediated by complement inhibition. Studies using human samples confirmed our observations in APS-mice. Diminished levels of C5a were observed in patients after treatment with HCQ.

It has been described that IgG purified from patients with obstetric APS inhibits trophoblast invasion *in vitro* in a TLR4-dependent manner [49] and that HCQ decreases TLR4 mRNA in BeWo's cells restoring trophoblast invasion [50]. Because C5a can modulate TLR signalling through a receptor-mediated mechanism [51], it is possible that downregulation of TLR4 might act synergistically with complement inhibition in the protective effects of HCQ in a $\beta_2$ GPI-induced pregnancy morbidity.

Our studies suggest that HCQ is an effective therapy not only to prevent fetal death and placental insufficiency but also to protect the fetal brain in APS.

Excess complement generation has been associated with significant thrombophilia in numerous pathologies including APS [52]. That HCQ inhibits complement activation might also explain the beneficial effects of this drug to prevent thrombotic events in patients with APS [53].

## **ACKNOWLEDGEMENTS**

This work was supported by TheirWorld for the Jennifer Brown Research Lab. This study also received support from the NIHR Biomedical Research Centre based at Guy's and St Thomas' NHS Foundation Trust and King's College London. The views expressed in this publication are those of the author and not necessarily those of the NHS, the NIHR, or the Department of Health. SPECT/CT scanning equipment at KCL was funded by an equipment grant from the Wellcome Trust (grant number WT

084052/Z/07/Z). The authors want to thank Margaret Cooper for her help in the radiolabelling of the autoantibodies.

## **AUTHORSHIP CONTRIBUTIONS**

LB and GC performed experiments, GG designed the research, performed experiments, analysed data and wrote the manuscript, GS collected human samples for the Registry of Connective tissue diseases (10/H0405/35) at St Thomas' Hospital, KS, MM performed CT/SPECT and ex vivo gamma counting studies, RL performed and analysed the spectroscopy data, PJB contributed to the analysis of SPECT/CT data.

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**TABLE 1**

	Sex/age	ACA-IgG	$\beta_2$ GPI-IgG	LA	diagnostic	Clinical features	Obstetric APS
Pt 1	F/52	+	+	+	PAPS	arterial thrombosis	RM Stillbirth
Pt 2	M/62	+	+	+	PAPS	DVT, stroke	
Pt 3	F/48	+	+	+	PAPS	DVT, PE	Preeclampsia
Pt 4	F/38	+	+	+	PAPS	DVT	RM preeclampsia
Pt 5	M/54	+	+	+	PAPS	arterial and venous thrombosis	
Pt 6	F/54	+	+	+	PAPS	DVT	RM, preterm birth, stillbirth
Pt 7	F/45	+	+	+	PAPS		RM, preeclampsia
Pt 8	F/38	+	+	+	PAPS		RM Stillbirth
Pt 9	F/31	+	+	+	PAPS	DVT	RM IUGR
Pt 10	F/34	+	+	+	PAPS		Preeclampsia HELLP syndrome
Pt 11	F/32	+	+	+	PAPS		RM preeclampsia
Pt 12	F/34	+	+	+	PAPS	venous thrombosis	RM, preeclampsia

**TABLE 2**

	<b>FRF(%)</b>	<b>Placenta (mg)</b>	<b>Fetus (mg)</b>
<b>NHIgG</b>	10.4±5.9	122±9.9	401.5±22.8
<b>APS</b>	54.2±11.1*	87.5±9.6*	302.1±18.5*
<b>APS +HCQ</b>	11.0±5.7	115.8±11.3	385.1±21.5
<b>HCQ</b>	10.9±1.0	131±15.3	398.3±19.5

**TABLE 3**

	Placenta				Fetal brain			
	Glc/Cr	Lac/Cr	PCh/Cr	GPC/Cr	Glc/Cr	Lac/Cr	PCh/Cr	GPC/Cr
<b>NHIgG</b>	1.04 ±0.40	4.95 ±0.91	1.69 ±0.73	0.82 ±0.36	0.28 ±0.14	1.61 ±0.9	0.75 ±0.13	ND
<b>APS-mice</b>	0.058 ±0.005*	0.54 ±0.07*	0.50 ±0.19*	0.29 ±0.19*	0.058 ±0.005*	0.32 ±0.13*	0.35 ±0.08*	ND
<b>APS- mice + HCQ</b>	1.105 ±0.32	4.23 ±0.78	1.61 ±0.61	0.92 ±0.43	0.27 ±0.09	1.65 ±0.85	0.81 ±0.09	ND

**TABLE 4**

	Pregnant			Non pregnant	
In <sup>111</sup> -conjugated IgG (ng IgG/g tissue)	a $\beta_2$ GPI	a $\beta_2$ GPI +HCQ	NHIgG	a $\beta_2$ GPI	NHIgG
Blood	89±27*	98±22*	304±42	261±110#	178±103#
Placenta	500±245	490±154	615±312		
Fetal brain	397±71*	427±60*	113±98		
Spleen	273±56	285±90	226±54	809±180#	572±167#
Liver	192±91	178±105	154±43	521±163#	372±65#

**TABLE 5**

	OD $\lambda$ 450 nm x 100		C3adesArg (ng/ml)
	C1q binding	C3b/iC3b/C3c	
FD1 (control)	65 $\pm$ 08	17 $\pm$ 5	97 $\pm$ 23
FB1 (complement activating antibody)	568 $\pm$ 47*#	102 $\pm$ 12*#	892 $\pm$ 54*#
FB1+ HCQ20	392 $\pm$ 67*#	81 $\pm$ 9*	556 $\pm$ 28*#
FB1+HCQ50	197 $\pm$ 45*#	38 $\pm$ 11*#	235 $\pm$ 45*#
FB1+HCQ100	57 $\pm$ 12#	15 $\pm$ 8#	86 $\pm$ 19#
FB1+serum from HCQ-treated mouse	48 $\pm$ 14#	21 $\pm$ 6#	78 $\pm$ 21

**TABLE 6**

	<b>C3a (ng/ml)</b>	<b>C5a (ng/ml)</b>
<b>NHIgG</b>	43±7	21±5
<b>APS</b>	156±34*	142±31*
<b>APS + HCQ</b>	57±21	35±11
<b>HCQ</b>	39±9	18±7



**TABLE 7**

	Before HCQ			After HCQ		
	aβ <sub>2</sub> GPI (Units)	aCL (Units)	C5a (ng/ml)	aβ <sub>2</sub> GPI (Units)	aCL (Units)	C5a (ng/ml)
	IgG			IgG		
Patient 8	65	68	132	72	72	45
Patient 9	78	86	156	83	91	65
Patient 10	124	132	97	130	128	22
Patient 11	85	42	123	76	51	31
Patient 12	135	56	198	145	67	39
Mean ± SD	97±30	77±35	141±38	101±34	82±30	40±16*

## TABLE AND FIGURE LEGENDS

**Table 1.** Clinical and laboratory features of the patients used as a source of human  $\alpha\beta_2$ GPI antibodies (Pt1 - Pt7) and patients that received HCQ treatment (Pt 8 - Pt12). Pt indicates patient. All patients met Sidney Laboratory criteria for APS [28]. ACA= anticardiolipin,  $\beta_2$ = $\beta_2$ glycoprotein I ( $\beta_2$ GPI), RM=recurrent miscarriages, APS= primary antiphospholipid syndrome, DVT=deep vein thrombosis, PE= pulmonary embolism.

**Table 2.** Pregnancy outcomes in mice treated with NHlgG, APS-mice, APS-mice treated with HCQ and mice treated with HCQ alone. Fetal resorption frequency (FRF) was calculated as the number of resorptions divided by the total number of resorptions plus viable fetuses. N=6-7 / experimental group. \* different from NHlgG,  $p<0.05$

**Table 3.** *In vivo*  $^1$ HMRS studies in placenta and fetal brain.

Values of different different metabolic peaks in placentas and fetal brains of APS-mice with and without HCQ treatment and control mice treated with NHlgG at day 15 of pregnancy. The  $^1$ HMRS signal from creatine (Cr, measure of the global brain or placental function or integrity) was used to normalize the signals from metabolites measured within the same voxel. N=5-6 / experimental group. Glucose = Glc, lactate = Lac, glycerophosphocholine =GPC, phosphocholine =PCh. \* different from NHlgG,  $p<0.05$

**Table 4.** Ex-vivo gamma counting showing the biodistribution of NHlgG and  $\alpha\beta_2$ GPI in pregnant and nonpregnant mice. Results are expressed as amount of IgG per g of

tissue (ng IgG/ g of tissue) N=4-5 mice/group. \* different from NHlgG,  $p<0.05$ , # different from pregnant mice,  $p<0.05$

**Table 5.** Effect of HCQ on antibody complement C1q fixation and C3 activation capacity. Values of C1q fixation and C3b/iC3b/C3 generation are expressed as O.D. at wavelength ( $\lambda$ ) = 450. Complement fixing mouse monoclonal aPL antibody FB1 and control non fixing FD1 antibody were used in the ELISA. N=5-7 / experimental group.\* different from control antibody FD1,  $p<0.05$ ; # different from complement fixing antibody FB1,  $p<0.05$ .

**Table 6.** HCQ inhibits complement activation *in vivo*.

Complement split products C3adesArg and C5adesArg in serum from NHlgG-treated mice, APS-mice and APS-mice treated with HCQ. N=5-6 mice/experimental group.

\*Different from NHlgG,  $p<0.05$

**Table 7.** C5adesArg levels and anti $\beta_2$ -GPI titres in patients before and after (>60 days) treatment with HCQ (6.5 mg/kg/day). \* different from before HCQ,  $p<0.05$

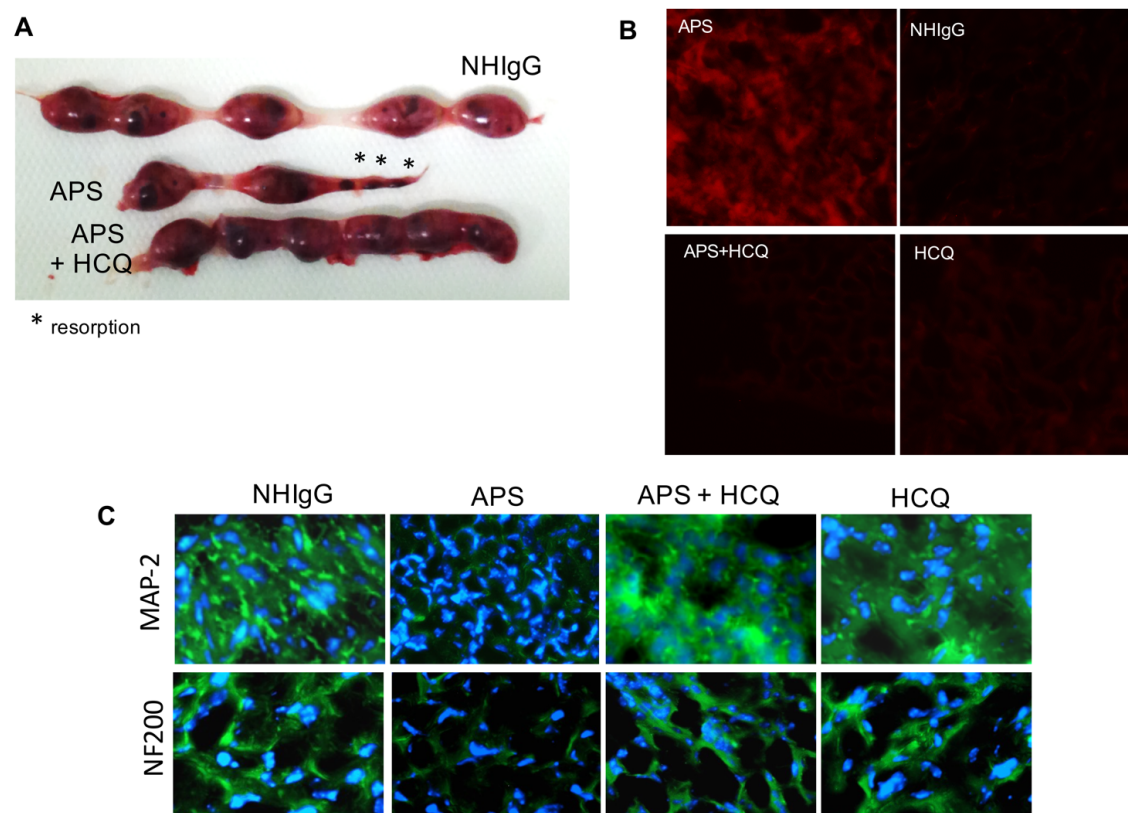
**Figure 1.** *Protective effects of HCQ in pregnant treated with a $\beta_2$ GPI antibodies*

A- Uteri harvested at day 15 of pregnancy. There were increased number of resorptions (asterisks) in the utero of APS-mice compared to control mice treated with NHlgG and APS- mice treated with HCQ. Data are representative of observations in 5–6 mice per group.

B- Increased superoxide production (DHE, red staining) in APS-mice compared to NHIgG-treated mice. HCQ treatment prevented increased superoxide production induced by  $\alpha\beta_2$ GPI antibodies (n=5-6 mice per group).

C- Microscope photomicrographs demonstrating decreased NF-200 and MAP-2 staining (green fluorescence) in the fetal brains from APS-mice compared to NHIgG control group. HCQ treatment prevented abnormal cortical brain development and increased NF-200 and MAP-2 staining. Diamidino-2-phenylindole (DAPI) was used for nuclear counterstain in immunofluorescence studies in C and D.

Microphotographs represent one of 5 similar experiments.



**Figure 2:** NanoSPECT/CT images of indium<sup>111</sup>-labelled  $\alpha\beta_2$ GPI and NHIgG

A- Non pregnant mouse injected with  $\alpha\beta_2$ GPI (left) and NHIgG (right)

B- Pregnant mouse injected with  $\alpha\beta_2$ GPI (left) and NHIgG (right)

B1- SPECT/CT of isolated utero from a mouse injected with In111- $\alpha\beta_2$ GPI

B2- SPECT/CT of isolated utero from a mouse injected with In111-NHIgG

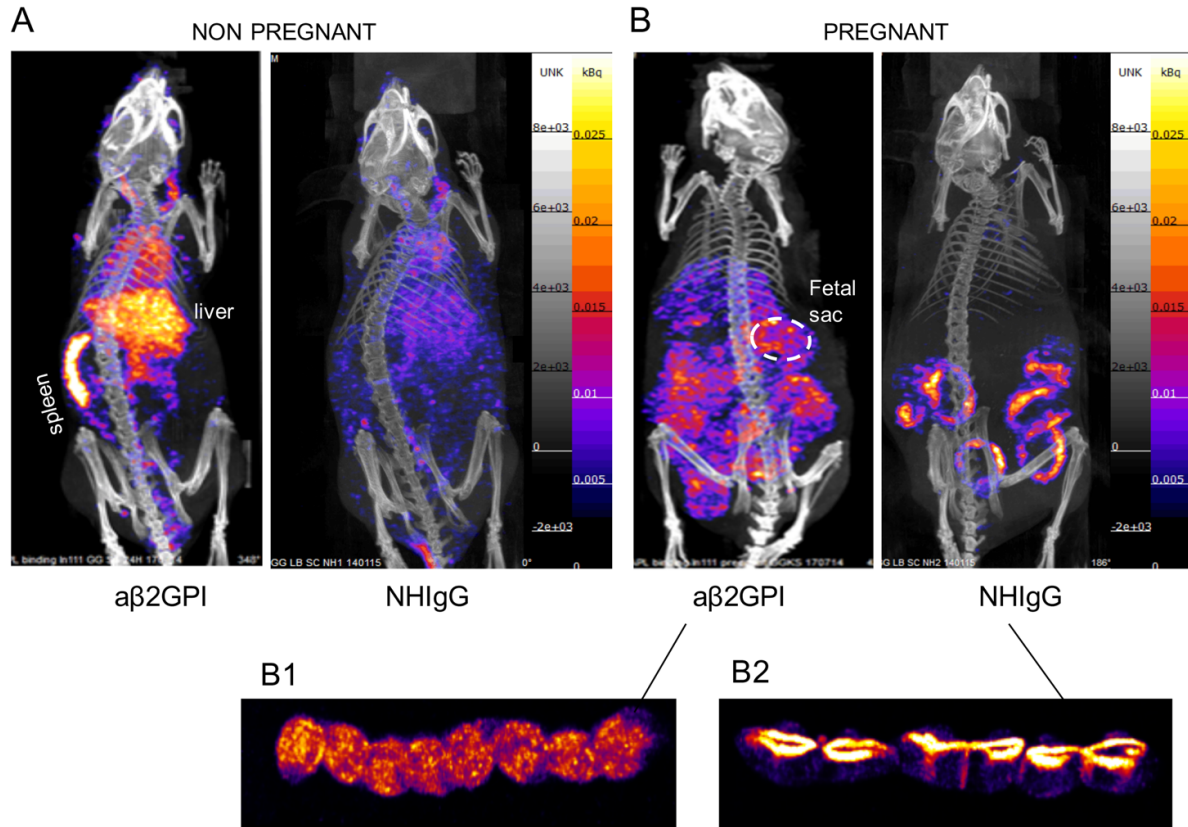


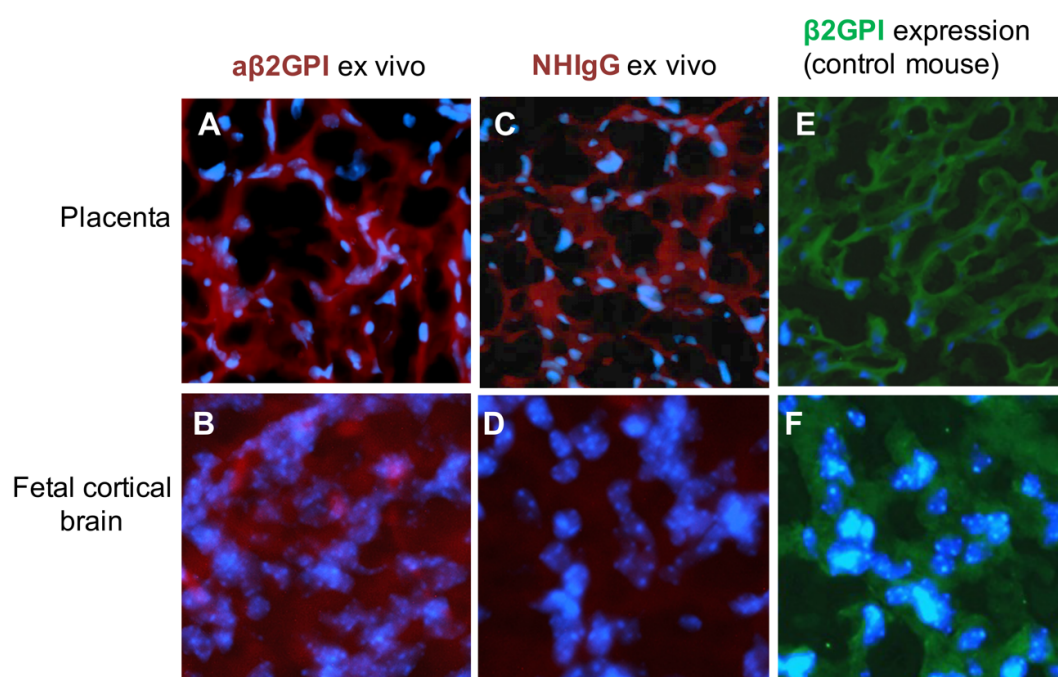
Figure 2

**Figure 3.** Immunohistochemical studies to detect deposition of  $\alpha\beta_2$ GPI and NHIgG in placenta and fetal brain. Expression of  $\beta_2$ GPI protein in placenta and fetal brain in control mice.  $\alpha\beta_2$ GPI and NHIgG deposition in mouse tissue (placenta and fetal cortical brain) was detected using an antihuman IgG antibody labelled with Texas Red.

A- detection of  $\alpha\beta_2$ GPI in the placenta from mouse injected  $\alpha\beta_2$ GPI. B- Detection of  $\alpha\beta_2$ GPI in fetal cortical brain from a mouse injected with  $\alpha\beta_2$ GPI. C- Detection of NHIgG in the placenta from a mouse injected with NHIgG. D- Detection of NHIgG in the fetal cortical brain in a mouse injected with NHIgG.

Expression of  $\beta_2$ GPI in placenta (E) and fetal brain (F) was detected with FITC-conjugated antibodies (green fluorescence). Diamidino-2-phenylindole (DAPI) was used for nuclear counterstains in all immunofluorescence studies. Data are representative of observations in 5–6 mice per group. 10 views per slide were analyzed in each experimental condition.

Figure 3



**Figure 4.** *HCQ does not affect aβ2GPI antibodies binding*

A- Deposition of aβ2GPI antibodies (red fluorescence) in placenta and fetal brain in APS-mice and APS-mice treated with HCQ.

B- C3 deposition (green fluorescence) in placentas and fetal brains from APS-mice and APS-mice treated with HCQ. C3 deposition was attenuated by HCQ treatment both in placentas and fetal brains at d15 of pregnancy in APS-mice.

Diamidino-2-phenylindole (DAPI) was used for nuclear counterstains in all immunofluorescence studies. Data are representative of observations in 5–6 mice per group.

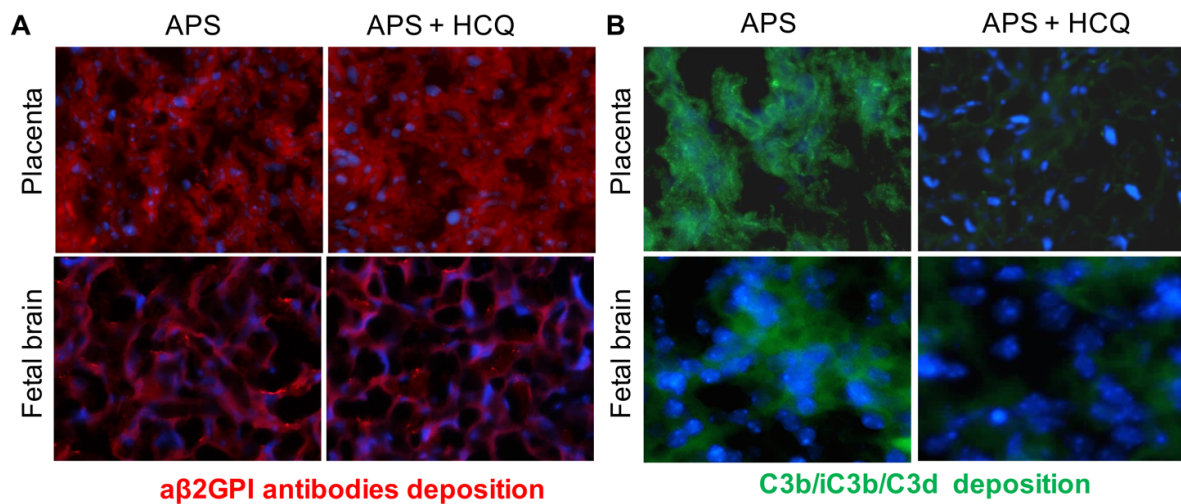


Figure 4

**Supplementary file** for online publication. Video showing nanoSPECT/CT images of indium<sup>111</sup>-labelled  $\alpha\beta_2\text{GPI}$  and NHIgG in pregnant and non pregnant mice.